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Data Selection and Treatment of Chemicals Tested for Genotoxicity and Carcinogenicity

by Nicola Loprieno,* Guido Boncristiani,* Gregorio Loprieno,* and Marco Tesoro*

A database containing qualitative and quantitative results of experimental studies in the fields of genotoxicity and carcinogenicity has been developed. By analyzing results of the studies performed by the U.S. National Toxicology Program, or by a similar program developed in Japan, or reported in the scientific literature, as well performed by private organizations, information has been collected relating to 3389 chemicals, identified by their CAS number. The studies considered for the database include three genotoxicity/mutagenicity short-term test (STTs), namely, two *in vitro* (Salmonella, gene mutation assay, and mammalian cells/human lymphocytes chromosome aberration assay) and one *in vivo*, the rodent bone marrow micronucleus assay. To investigate the possible predictive value of these STT assays for carcinogenicity, the results of animal long-term bioassays have also been collected. We have re-evaluated all the genotoxicity studies and the majority of those cases studied in different laboratories with contrasting results has been resolved; a small proportion of questionable cases is, however, still present in the database. In total, 2898 (85.5%) of the chemicals have been tested in the Salmonella assay; 1399 (41.3%) have been tested in the *in vitro* chromosome aberration assay; 319 (9.4%) have been tested in the *in vivo* rodent bone marrow cell micronucleus assay; 716 (21.2%) of the chemicals have been tested in the *in vivo* animal long-term bioassay. For 1118 chemicals tested in the Salmonella assay, 30,650 quantitative studies have been included in the database, thus allowing a possible classification of mutagenic chemicals according to their mutagenic potency. One thousand nine hundred chemicals (56.1%) have shown positive results in at least one of the four different assays, thus leaving 1,489 chemicals (43.9%) with negative results.

By analyzing the correlation between genotoxicity, as shown by the three STTs considered, and carcinogenicity, we have demonstrated that the positive predictivity increases to a value of 95.6% if the three STTs are considered together (two *in vitro* and one *in vivo* STT); similarly, the negative predictivity rises to a value of 89.6% with the same three assays. The accuracy, or the concordance, of the STT results and the carcinogenicity results was 92.5% for the three STTs. Although the results collected are of high interest for scientific and practical actions, the aim of the present study is to prepare a genotoxicity/carcinogenicity database for a further quantitative structure–activity relationship (QSAR) study based on a computer chemistry analysis.

Introduction

The assessment of genotoxic effects of chemicals may be performed by means of a series of assays based on a variety of biologic test systems. *In vitro* and *in vivo* assays are available to detect either one of three major types of irreversible damage in genetic material, namely, gene mutation, chromosomal aberration, and DNA damage and repair. The induction of such genotoxic effects is indicative also of potential carcinogenicity of at least one large class of chemicals (genotoxic carcinogens); also short-term tests (STTs) are extensively performed for screening large numbers of chemical substances to identify those chemicals that should have priority for long-term *in vivo* studies needed for carcinogenicity assessment. However, the choice of genetic end points or the choice of biologic systems, and/or the combinations of end points and systems that might be more adequate for distinguishing carcinogens from noncarcinogens is still a matter of debate, and different tiered testing approaches of STT batteries are suggested for assessing potential carcinogenicity of chemical substances.

In the current practice, such assessment relies on qualitative examination of responses obtained in a number of tests, which are generally selected by experts on a case-by-case basis and are not necessarily the same tests. This strategy, as it stands, is not devoid of some degree of subjectivity and might lead to some differences in predictions of carcinogenicity of the same chemicals. The strategy would be more efficient if quantification of the dependence of carcinogenicity from STT genotoxicity data were available.

In the context of evaluating chemical substances, the need for relying on predictive methodologies is generally agreed upon. At present such methodologies are used on a qualitative basis for predicting a number of toxic end points. The rationale for these

*Dipartimento di Scienze dell'Ambiente e del Territorio, University of Pisa, Laboratory of Genetic Toxicology, Via S. Guiseppe 22, 56100 Pisa, Italy.

Address reprint requests to N. Loprieno, Dipartimento di Scienze dell'Ambiente e del Territorio, University of Pisa, Laboratory of Genetic Toxicology, Via S. Guiseppe 22, 56126 Pisa, Italy.

predictions basically stands on the experience that *a*) there are correlations between the structure of the molecules and their properties, including biologic activities and toxic effects and *b*) there are toxic effects that are correlated to other toxic effects. This strategy would be more efficient if quantification of these correlations could be made; in other words, if mathematical models could be developed to define the dependence of a given toxic effect from either the molecular structure (referred to as quantitative structure–activity relationship, QSAR) or other toxic effects (referred to as quantitative activity–activity relationship, QAAR). These two approaches are complementary to each other, especially in the area of genotoxicity. Whereas single genotoxic end points, or a combination of them, may be modeled by QSAR, carcinogenicity may tentatively be modeled by QAAR, under the assumption that carcinogenic activity may be predicted on the basis of responses obtained in suitable combination of mutagenicity data.

Computer modeling of toxicity is a suggestive task, though not a simple one. It is not difficult to compile data tables, run them through some computer packages, and end up with a model, but criteria used in the compilation of data, the quality of the data, and the method of data analysis can hide weaknesses, sometimes difficult to perceive, that may render the predictive power of the model questionable.

To make toxicity models acceptable substitutes of toxicity testing many things are requested. These include proper use of the state of the art in a number of branches of the disciplines involved (chemistry, toxicology, and statistics) and adequate data. Toxicity of chemicals is related to complex phenomena so that fundamental models based on *ab initio* calculations are, at present, inadequate. Vice versa, statistical models based on the principle of analogy such as QSARs may work. A number of conditions must, however, be met by the database, and the data analytic method must also be used. One of the most important conditions to take into account is the quality of the data used in the analysis.

The relevance of the present studies on the QSAR resides also on several other factors: *a*) the need for different government agencies such as the U.S. EPA to define the toxicologic studies to be carried out on the new chemical substances and to be notified under the Toxic Substances Control Act (TSCA) legislation; *b*) the need to predict with high probability the toxicologic hazard of those numerous substances present on the market for which there might be an emergency faced by public authorities; *c*) the need to know how many studies are requested for defining the hazard related to an unknown chemical substance; *d*) the need to identify the toxicological mechanism by which a great number of substances produce different types of biological adverse effects; and *e*) the need to provide the public with more realistic conclusions on the benefit–risk evaluation for a given chemical substance of large use.

The QSAR studies are particularly interesting in the field of mutagenesis and carcinogenesis due to their partial overlapping and the irreversible nature of their biological implications. For a long time attempts have been made to employ the mutagenicity short-term studies to predict the carcinogenic potential activity of the chemical substances, not only for biological considerations, but also for economic reasons, if one considers the cost of the long-term studies.

According to the International Agency for Research on Cancer of Lyon (IARC-WHO), in 1990 there were 732 chemical substances that had been tested for carcinogenicity through adequate studies on animals: 85% of them have proven to be carcinogenic. In contrast, the literature reports that almost 10,000 chemical substances have been tested for mutagenicity. For this reason it is quite simple to understand why mutagenicity studies (most of them using *in vitro* methodologies) have been and are being carried out and why the results are used to develop mathematical models to predict the potential carcinogenic activity of mutagenic molecules. For molecules of new chemicals such studies attempt to assess toxicological potential.

On the basis of independent systems that make use of the chemical structure of substances, the presence of structural genotoxic alert fragments, or computerized evaluation systems, such as CASE, COMPACT, etc., several researchers have developed a prediction hypothesis on the possible different results that might be produced by the long-term carcinogenicity studies currently carried out on 44 chemical substances by the National Toxicology Program (*1–4*).

Our objective is to develop a predictive model for genotoxicity. In the present paper we have assumed as a basis of this objective the collection and the evaluation of at least two series of testing procedures recognized as indicative of the genotoxicity property of a chemical. These two methodologies are represented by *a*) the *Salmonella typhimurium* reverse mutation assay for analyzing the ability to induce molecular (gene) mutations in the genetic material and *b*) the chromosome aberration-mammalian cell growth *in vitro* assay for analyzing the ability to induce structural (chromosomal) mutations in the genetic material that is organized in chromosomes in the cell nucleus. These two methodologies, on the basis of their experimental procedures, represent the most suitable technical approach to maximize the cell exposure to a chemical solution, which is a basic condition for a chemical to enter into a cell structure and to react with the DNA (genetic) material, if it is a genotoxic agent.

Ashby (*5*) stated, on the basis of experimental data, that the genotoxicity of a chemical can be adequately defined using a

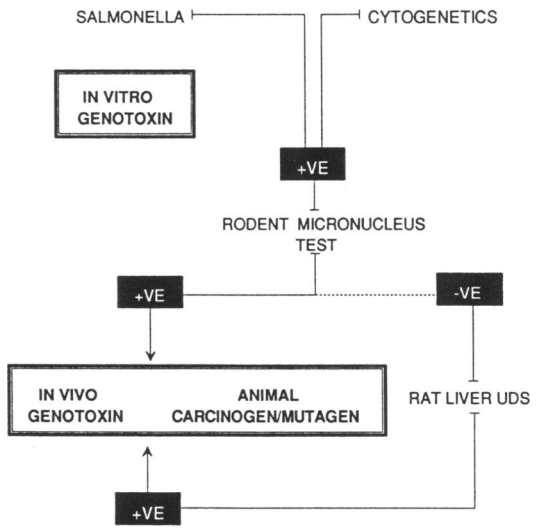

FIGURE 1. Proposed genotoxicity short-term tests tiered scheme for predicting carcinogenic effect.

combination of the Salmonella mutation assay and one for the assessment of chromosome aberrations *in vitro*. The indication that a chemical is positive in these two *in vitro* assays clearly defines what is today known as an "*in vitro* genotoxin." Because it has be shown that not all *in vitro* genotoxins are carcinogenic to mammals, it has been recommended that all newly discovered *in vitro* genotoxins should be assessed *in vivo* using very few additional tests (Fig. 1). The experimental data have shown clearly the weight of evidence resulting from the application of two very simple *in vitro* genotoxicity assays in the evaluation of the mutagenic potential of the chemicals. Our conclusion is that these types of assays could well represent the basis for the correct classification of a "genotoxin" and that these data should be used for discriminating a mathematical model for predicting genotoxicity of chemical substances.

In addition to collecting available data, in this preliminary analysis we have also made an attempt to define the possible correlations existing between *in vitro* STT results obtained with the two tests mentioned above and *in vivo* results by applying the previous hypothesis (*5*). For the *in vivo* test, we have chosen the rodent bone marrow micronucleus assay because considerable data exist that make the evaluation of these correlations possible. Moreover, the collection of results derived from long-term carcinogenic tests performed on chemicals has allowed us to make an attempt to investigate the predictive value of the STTs for carcinogenicity.

Materials

A database for a QSAR study includes a number of chemical substances and, for each of them, a number of numerical descriptors of the molecular structure (x-data) and a number of measured biological responses (y-data). Geneticists are aware of the fact that a single genetic end point is insufficient to evidentiate the genotoxic profile of a chemical because a variety of genetic toxic effects and impairments of genetic material processes may lead to an irreversible change in the genetic structure of an organism.

The literature provides material for our present analysis that could not always be used for developing a model. The data reported in the literature, when these three assays have been used (this applies also to other genotoxicity assays) are extremely variable for a number of reasons: *a*) the data are presented only graphically; *b*) different protocols have been used; *c*) a maximum dose for the analysis has not always been applied, especially in the negative results; *d*) a replication of the experiment is not present in many studies; *e*) positive and negative controls rarely have been reported; and *f*) criteria for defining a positive series of results are different in different laboratories. For these reasons we have proceeded to a particular selection of the data to be used in the present study.

From the analysis of literature data, we have selected sets of studies for their intrinsic homogeneity. These sets of data are:

1. The National Toxicology Program (NTP) developed by the U.S. Department of Health and Human Services as a cooperative effort to strengthen and coordinate research and testing on toxic chemicals, established in 1978. Reports containing the results of all studies conducted by the NTP have been published (*6–12*). The quantitative results of the NTP-developed STTs have been reported in the literature (*13–22*). In its Cellular and Genetic Toxicology Program, the NTP is involved in development, improvement, and validation of STTs for mutagens and carcinogens, using STTs to detect and characterize chemicals that may pose carcinogenic or genetic risks to humans. The NTP is focused on developing and validating *in vitro* and *in vivo* systems for determining the genotoxicity of chemicals. In the Annual Plan of the fiscal year 1988 (*11*), the NTP reports that "testing with Salmonella strains has been completed on a total of 1566 samples and 1190 unique chemicals since the initiation of the testing program."

   The NTP has developed a database of STT results, created by using chemicals tested for carcinogenicity by the National Cancer Institute and the NTP. This database allows for the evaluation of the Salmonella assay and several other STTs with respect to their ability to predict carcinogenesis or other short-term assay results. Although no assay can detect all carcinogens, in this database a positive result in Salmoella was a better predictor of carcinogenicity in rodents than a positive result in other assays. The Salmonella assay differed markedly in its response to chemicals of different classes.

   Chemicals that are carcinogenic only in rats or mice were tested to determine to what extent their mutagenicity depends on the mouse or rat S-9 activation system. There does not appear to be any correlation between the species specificity of the carcinogens and the rodent liver S-9 requirements for mutagenicity. This finding has implications for the use of mutagenicity results for predicting carcinogenicity. A similar testing program, although not at the same level of quantitative development, is conducted under NTP on the evaluation of the cytogenetic damage induced in mammalian cells *in vitro*.
2. The Institute for Future Technology, Japan, Cooperative Program on Long-Term Assays for Carcinogenicity (*23*). This program includes data on Salmonella, as well on mammalian cells for chromosome aberration. The Salmonella data have been originated in several laboratories; the *in vitro* chromosome aberration results have been originated in only one laboratory and they have been reported in publications by Ishidate in 1983 (*24*) and in 1988 (*25*).
3. Other Salmonella results or chromosomal aberration results have been collected from selected papers in the scientific literature (*26–33*).
4. The data on the micronucleus assay have been collected from either a paper by Ishidate et al. (*26*), from Hayashi et al. (*32*), or from private studies.

From the available results, we have collected the data for the present analysis presented in Table 1. Several attempts were made to classify the "questionable" mutagens or nonmutagens by reanalyzing the experimental data to draw our conclusion. In several cases our conclusion was definitive for the classification of a chemical based on a more critical review of the available data.

Table 1. Data collected for analysis.

Category	Number
Chemicals tested in *Salmonella typhimurium* for gene mutation	2898
Chemicals tested in *in vitro* mammalian cells (animal tissue culture, Chinese hamster ovary, or human lymphocytes) for induction of chromosomal aberration	1399
Chemicals tested *in vivo* in bone marrow cells for induction of micronuclei	319
Chemicals tested in Salmonella gene mutation and mammalian cell chromosomal aberration	1053
Chemicals tested in *in vivo* assays for assessment of carcinogenic potential	716
Total chemicals for which data were collected	3389

Results and Discussion

The overall results are reported in Figure 2; for each type of assay the numbers of chemicals classified positive, negative, or questionable are reported. Among the chemicals tested in the two *in vitro* STTs (Salmonella and chromosome aberrations) the database includes 1053 chemicals that have been tested in both assays; for these chemcials, the results of the different combinations are reported in Figure 3. Moreover, 270 chemicals have been tested in the two *in vitro* STTs, as well in the *in vivo* rodent bone marrow micronucleus test: that data are reported in Figure 4. One or both the *in vitro* STTs with negative results indicate a chemical with a low probability (25–15%) of producing a positive result in the *in vivo* micronucleus test. This probability rises to about 50% if the results from both the *in vitro* STTs are positive (Fig. 5).

When comparing the results observed in one single STT or in a combination of dirrerent STTs with those observed in the carcinogenicity study, their accuracy varies according to a specific combination: the data of such a calculation are reported in Table 2. The accuracy values observed when the results from Salmonella or chromosome aberrations are considered more than 60% as observed by other authors (*18*). The inclusion of the *in vivo* micronucleus assay in a combination with the two STTs allows the accuracy value to raise to more than 80%. The best performance of the STTS for predicting the carcinogenicity is that one observed when the two *in vitro* STTS (Salmonella and chromosomal aberrations) are combined with the *in vivo* micronucleus assay; in this case a 92.5% value of the accuracy has been calculated (Fig. 6). In Table 3 the positive and the negative predictivity of STTs for carcinogenicity has been calculated and reported.

The inclusion of an *in vivo* STT in a battery of genotoxicity STTs, such as the *in vivo* rodent bone marrow cells micronucleus assay, represents an improvement in the present strategy of using STTs to prescreen the potential carcinogenic compounds. Specific data and results on this category of chemical susbstances will be reported elsewhere (N. Loprieno, in preparation).

CHEMICALS	MUTAGENICITY			CARCINOGENICITY
	Salmonella	Chrom. Ab.	Micronuclea	
	IN VITRO BIOASSAYS		IN VIVO BIOASSAYS	
TOTAL Nr.	2,898	1,399	319	716
%	85.5	41.3	9.4	21.2
POSITIVE	1,298	710	97	451
%	44.8	50.7	30.4	62.9
NEGATIVE	1,584	668	222	231
%	54.6	47.7	69.6	32.3
QUESTIONABLE	16	21	—	34
%	0.6	1.6		4.8

FIGURE 2. Classification of 3389 chemicals included in the database.

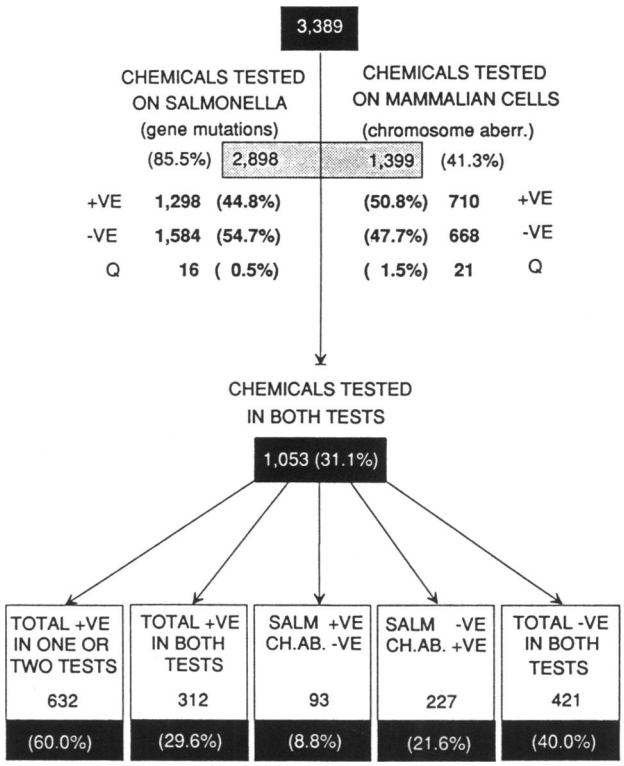

FIGURE 3. *In vitro* genotoxicity data on chemicals. +VE, positive; −VE, negative; Q, questionable.

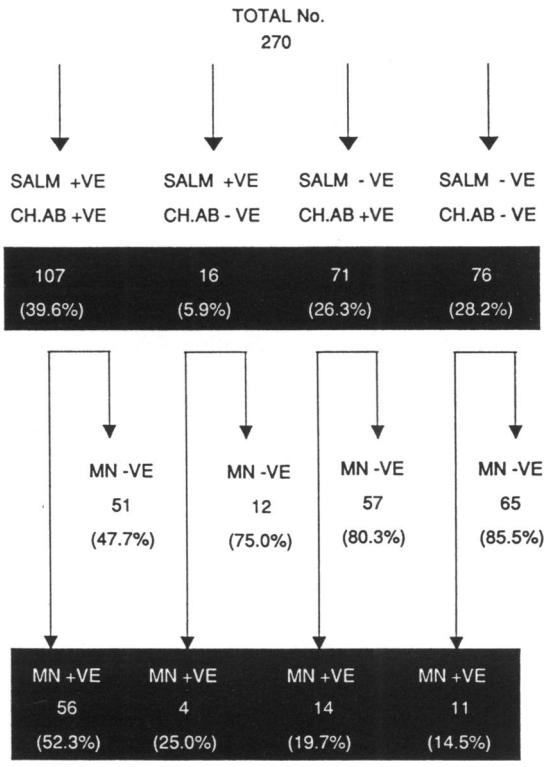

FIGURE 4. Chemicals tested for mutagenicity/genotoxicity in two *in vitro* assays and in one *in vivo* assay (micronucleus). SALM, Salmonella; CH.AB, chromosome aberrations; MN, micronucleus; +VE, positive; −VE, negative.

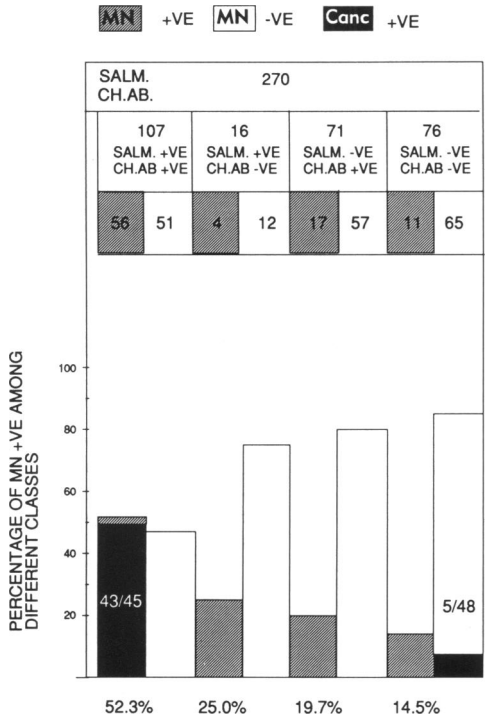

FIGURE 5. Prediction of *in vitro* and *in vivo* mutagens/carcinogens. SALM, Salmonella; CH.AB, chromosome aberration; MN, micronucleus; Canc, carcinogen; +VE, positive; −VE, negative.

Table 2. Accuracy of correct identification of chemicals.[a]

Short-term test	No. of chemicals tested for carcinogenicity: Total	Correct	Accuracy, %
Salmonella	544	373	68.6
Chromosome aberration	445	286	64.3
Micronucleus	163	115	70.6
Salmonella + chromosome aberration	310	222	71.6
Salmonella + micronucleus	113	96	85.0
Chromosome aberration + micronucleus	107	94	87.9
Salmonella, chromosome aberration, and micronucleus	93	86	92.5

[a]Accuracy: with tested population of chemicals is the proportion of chemicals correctly identified as carcinogens or noncarcinogens by the test and is calculated: number of correct test results/number of chemicals tested. Concordance: the percentage of qualitative agreements between short-term tests and rodents carcinogenicity test results (*18*).

In the present analysis we found 11 chemicals that were classified negative in Salmonella and chromosome aberration assays but positive in the *in vivo* micronucleus assay (Fig. 4). These chemicals are reported in Table 4. Five chemicals out of the 11 have been classified also as animal carcinogens. Our opinion is that with these chemicals we are dealing with a specific class of compounds, such as trichloroethylene, vincristine, toluene, and chlorobenzene for which the mechanism of genotoxicity could not be fully applied. We are at present collecting more information on this class of chemicals.

The databases developed as reported in this paper represents an adequate resource for developing quantitative structure–activity relationships.

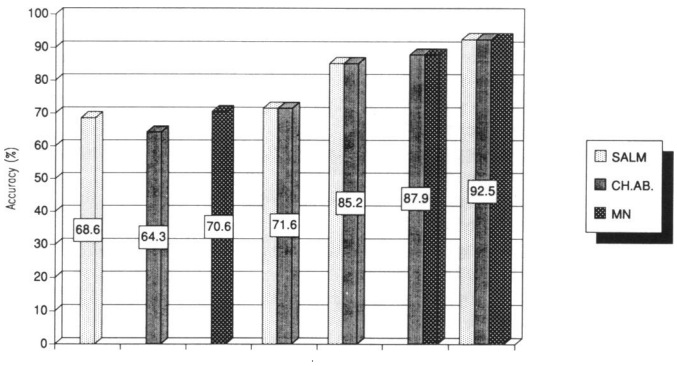

FIGURE 6. Accuracy of correct identification of carcinogenic/noncarcinogenic chemicals. SALM, Salmonella; CH.AB, chromosome aberration, MN, micronucleus.

Table 3. Predictivity of carcinogenicity and noncarcinogenicity of short-term tests.

Short-term test	Predictivity: Positive, %		Negative, %	
Salmonella	63.9	(216/338)	76.2	(157/206)
Chromosome aberration	67.2	(178/265)	60.0	(108/180)
Micronucleus	56.8	(54/95)	89.7	(61/68)
Salmonella + chromosome aberration	82.7	(129/156)	61.6	(93/151)
Salmonella + micronucleus	95.7	(44/46)	77.6	52/67
Chromosome aberration + micronucleus	92.3	(48/52)	83.0	(46/55)
Salmonella, chromosome aberration, and micronucleus	95.6	(43/45)	89.6	(43/48)

Table 4. Chemicals negative in the *in vitro* genotoxicity test and positive in the micronucleus test.

Chemical	CAS no.	Ames	Chromosome aberration	Micronucleus	Cancer	Species
Chlorobenzene	108-90-7	−	−	+	+	Rats
o-Dichlorobenzene	95-50-1	−	−	+	+	Rats, mice
p-Dichlorobenzene	106-46-7	−	−	+	+	Rats, mice
Isoxaben	82558-53-7	−	−	+		
Toluene	108-88-3	−	−	+	+	Rats
1,3,5-Trichlorobenzene	108-70-3	−	−	+		
1,2,4-Trichlorobenzene	120-82-1	−	−	+		
1,2,3-Trichlorobenzene	87-61-6	−	−	+		
Trichloroethylene	79-01-6	−	−	+	+	Rats, mice
Trimethoprim	738-70-5	−	−	+		
Vincristine	57-22-7	−	−	+		

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